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(54) Title: <b>PHOSPHONYLDIPEPTIDES USEFUL IN THE TREATMENT OF CARDIOVASCULAR DISEASES</b>			
(57) Abstract  The present invention relates to isolated nucleic acid constructs containing a sequence encoding a <i>Myceliophthora</i> laccase, and the laccase proteins encoded thereby.			

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## Phosphonyldipeptides useful in the treatment of cardiovascular diseases

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### Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the  
10 purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, of a thermophilic ascomycete, *Myceliophthora*.

### 15 Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper-containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable  
20 phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and  
25 humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus*, *Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, and perfect forms of *Rhizoctonia*. Laccase  
30 exhibits a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have

found many potential industrial applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water treatment.

- Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ depending on the specific substrate. A number of these fungal laccases have been isolated, and the genes for several of these have been cloned. For example, Choi et al. (Mol. Plant-Microbe Interactions 5: 119-128, 1992) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus, *Cryphonectria parasitica*. Kojima et al. (J. Biol. Chem. 265: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch (Experientia 41: 801, 1985; PNAS USA 83: 8854-8858, 1986) have reported the cloning and partial sequencing of the *Neurospora crassa* laccase gene. Saloheimo et al. (J. Gen. Microbiol. 137: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*.
- Attempts to express laccase genes in heterologous fungal systems frequently give very low yields (Kojima et al., *supra*; Saloheimo et al., Bio/Technol. 9: 987-990, 1991). For example, heterologous expression of *Phlebia radiata* laccase in *Trichoderma reesei* gave only 20 mg per liter of active enzyme (Saloheimo, 1991, *supra*). Although laccases have great commercial potential, the ability to express the enzyme in significant quantities is critical to their commercial utility. At the present time there are no laccases which are expressed at high levels in commercially

utilized hosts such as *Aspergillus*. Thus, the need exists for a laccase which can be produced in commercially useful (i.e., gram per liter or more) quantities. The present invention fulfills such a need.

5

#### Summary of the Invention

The present invention relates to a DNA construct containing a nucleic acid sequence encoding a *Myceliophthora* laccase. The invention also relates to an isolated laccase  
10 encoded by the nucleic acid sequence. Preferably, the laccase is substantially pure. By "substantially pure" is meant a laccase which is essentially (i.e., ≥90%) free of other non-laccase proteins.

In order to facilitate production of the novel laccase,  
15 the invention also provides vectors and host cells comprising the claimed nucleic acid sequence, which vectors and host cells are useful in recombinant production of the laccase. The sequence is operably linked to transcription and translation signals capable of directing expression of  
20 the laccase protein in the host cell of choice. A preferred host cell is a fungal cell, most preferably of the genus *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the construct of the invention, or  
25 progeny thereof, under conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of  
30 phenolics is required. These processes include lignin manipulation, juice manufacture, phenol polymerization and phenol resin production.

#### Brief Description of the Figures

Figure 1 shows a restriction map of a 7.5 *EcoRI* fragment in pRMB1. The region hybridizing to the *N. crassa* laccase gene probe is shaded.

Figure 2 illustrates the nucleotide (SEQ ID NO: 1) and amino acid (SEQ ID NO: 2) sequence of *Myceliophthora thermophila* laccase. Lower case letters in the nucleotide sequence indicate the position of introns. Putative TATA and CAAT sequences in the promoter region are in boldface and underlined. Consensus lariat structures (PuCTPuAC) within the introns are underlined.

Figure 3 illustrates the construction of plasmid pRMB5.

#### Detailed Description of the Invention

*Myceliophthora thermophila* is a thermophilic Ascomycete originally described by Apinis (Nova Hedwigia 5: 57-78, 1963) and named *Sporotrichum thermophile*. Subsequent taxonomic revisions have placed this organism in the genus *Chrysosporium* (Von Klopotek, A. Arch. Microbiol. 98: 365-369, 1974) and later to *Myceliophthora* (Van Oorschot, Persoonia 9: 401-408, 1977). A number of organisms known by other names also appear to belong to this species. These include *Sporotrichum cellulophilum* (U.S. Patent No. 4,106,989); *Thielavia thermophila* (Fergus and Sinden, Can. J. Botany 47: 1635-1637, 1968); *Chrysosporium fergusi* and *Corynascus thermophilus* (Von Klopotek, *supra*). This species is known as a source of a number of different industrially useful enzymes, such as cellulases,  $\beta$ -glucosidase and xylanase (see, e.g., Oberson et al., Enzyme Microb. Technol. 14 : 303-312, 1992; Merchant et al., Biotechnol. Lett. 10: 513-516, 1988; Breuil et al., Biotechnol. Lett. 8: 673-676, 1986; Gilbert et al., Bioresource Technol. 39: 147-154, 1992). It has now been determined that *Myceliophthora* produces a neutral pH

laccase, and the gene encoding this laccase can be used to produce large yields of the enzyme in convenient host systems such as *Aspergillus*.

To identify the presence of a laccase gene in  
5 *Myceliophthora*, a 5' portion of the *Neurospora crassa* laccase gene (*lcc1*) is used as a probe, under conditions of mild stringency, in southern hybridization of total genomic DNA of different fungal species. An approximately 12 kb laccase specific sequence is detected in the *Myceliophthora*  
10 DNA. The *N. crassa* fragment is then used to screen about 20,000 plaques of an *M. thermophila* genomic DNA library in a  $\lambda$  EMBL4 bacteriophage cloning vector. Eight plaques strongly hybridize with the probe; from these eight, DNA is isolated from three. Each of these clones contains a 7.5 *Eco*RI  
15 fragment which also hybridizes to the probe (Figure 1). One of the fragments is subcloned into pBR322 to generate plasmid pRAMB1. Using the *lcc1* probe, the position of the coding region of the clone is determined. The entire *M. thermophila* coding region appears to be contained within a 3.2  
20 kb *Nhe*I-*Bgl*III segment, which is then cloned into pUC119 and sequenced by the primer walking method.

Once the sequence is determined, the positions of introns and exons within the gene is assigned based on alignment of the deduced amino acid sequence to the  
25 corresponding *N. crassa* laccase gene product. From this comparison, it appears that the gene (*lccM*) of *M. thermophila* is composed of seven exons (246, 79, 12, 70, 973, 69 and 411 nucleotides) interrupted by six introns (85, 84, 102, 72, 147, and 93 nucleotides). The coding region,  
30 excluding intervening sequences, is very GC-rich (65.5% G+C) and encodes a preproenzyme of 620 amino acids: a 22 amino acid signal peptide, a 25 amino acid propeptide, and a mature laccase comprising 573 amino acids. The sequence of

the *M. thermophila* gene and the predicted amino acid sequence is shown in Figure 2 (SEQ ID NOS: 1 and 2).

The laccase gene is then used to create an expression vector for transformation of *Aspergillus* host cells. The  
5 vector, pRAMB5 contains the *A. oryzae* TAKA-amylase promoter and terminator regions. The construction of pRAMB5 is outlined in Figure 3. *Aspergillus* cells are cotransformed with the expression vector and a plasmid containing the *pyrG* or *amdS* selectable marker. Transformants are selected on  
10 the appropriate selective medium containing ABTS. Laccase-producing colonies exhibit a green halo and are readily isolatable. Selected transformants are grown up in shake flasks and culture broths tested for laccase activity by the syringaldazine method. Shake flask cultures are capable of  
15 producing 0.2 or more g/liter of laccase, and in fermentors, yields of over 1-2 g/liter are observed.

According to the invention, a *Myceliophthora* gene encoding a laccase can be obtained by methods described above, or any alternative methods known in the art, using  
20 the information provided herein. The gene can be expressed, in active form, using an expression vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in a host cell  
25 independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and,  
30 optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be used



according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene, include but are not limited to the prokaryotic  $\beta$ -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the laccase DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*

$\alpha$ -amylase gene (*amyL*), the promoters of the *Bacillus*  
*stearothermophilus* maltogenic amylase gene (*amyM*), the  
promoters of the *Bacillus amyloliquefaciens*  $\alpha$ -amylase (*amyQ*),  
or the promoters of the *Bacillus subtilis* *xylA* and *xylB*  
5 genes. In a yeast host, a useful promoter is the *eno-1*  
promoter. For transcription in a fungal host, examples of  
useful promoters are those derived from the gene encoding *A.*  
*oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase,  
*A. niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase,  
10 *A. niger* or *A. awamori* glucoamylase (*glaA*), *Rhizomucor*  
*miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose  
phosphate isomerase or *A. nidulans* acetamidase. Preferred  
are the TAKA-amylase and *glaA* promoters.

15 The expression vector of the invention may also  
comprise a suitable transcription terminator and, in  
eukaryotes, polyadenylation sequences operably connected to  
the DNA sequence encoding the laccase of the invention.  
Termination and polyadenylation sequences may suitably be  
20 derived from the same sources as the promoter. The vector  
may further comprise a DNA sequence enabling the vector to  
replicate in the host cell in question. Examples of such  
sequences are the origins of replication of plasmids pUC19,  
pACYC177, pUB110, pE194, pAMB1 and pIJ702.

25 The vector may also comprise a selectable marker, e.g.  
a gene the product of which complements a defect in the host  
cell, such as the *dal* genes from *B. subtilis* or *B. li-*  
*cheniformis*, or one which confers antibiotic resistance such  
30 as ampicillin, kanamycin, chloramphenicol or tetracycline  
resistance. Examples of *Aspergillus* selection markers  
include *amdS*, *pyrG*, *argB*, *niaD*, *sc*, and *hygB*, a marker  
giving rise to hygromycin resistance. Preferred for use in

an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as  
5 described in WO 91/17243.

It is generally preferred that the expression gives rise to a product which is extracellular. The laccases of the present invention may thus comprise a preregion  
10 permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective  
15 preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the  $\alpha$ -factor from *Saccharomyces cerevisiae* or the calf  
20 preprochymosin gene. Particularly preferred, when the host is a fungal cell, is the preregion for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the maltogenic amylase form *Bacillus* NCIB 11837, *B. stearothermophilus*  $\alpha$ -amylase, or *Bacillus licheniformis* subtilisin. An effective signal  
25 sequence is the *A. oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal and the *Rhizomucor miehei* lipase signal.

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements,  
30 respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning, 1989).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombina-  
5 tant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more  
10 likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in  
15 connection with the different types of host cells.

The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus*  
20 *licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces*  
25 *murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The host cell may also be a eukaryote, such as  
30 mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces*

*cerevisiae*. Useful filamentous fungi may be selected from a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell.

- 5 Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of  
10 transforming *Fusarium* species is described by Malardier et al., 1989.

The present invention thus provides a method of producing a recombinant laccase of the invention, which method comprises cultivating a host cell as described above  
15 under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the  
20 invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

In a preferred embodiment, the recombinant production  
25 of laccase in culture is achieved in the presence of an excess amount of copper. Although trace metals added to the culture medium typically contain a small amount of copper, experiments conducted in connection with the present invention show that addition of a copper supplement to the  
30 medium can increase the yield of active enzyme many-fold. Preferably, the copper is added to the medium in soluble form, preferably in the form of a soluble copper salt, such as copper chloride, copper sulfate, or copper acetate. The final concentration of copper in the medium should be in the

range of from 0.2-2mM, and preferably in the range of from 0.05-0.5mM. This method can be used in enhancing the yield of any recombinantly produced fungal laccase, as well as other copper-containing enzymes, in particular  
5 oxidoreductases.

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed  
10 by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as  
15 determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following  
20 examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA  $\alpha$ -amylase promoter, and the *Aspergillus nidulans* *amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and used in co-transformation. The plasmid (or plasmids) is  
25 used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474, 1984).

Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid  
30 fragments specifically disclosed herein, for example, in Figure 1. It will also be apparent that the invention encompasses those nucleotide sequences that encode the same amino acid sequences as depicted in Figure 1, but which differ from the specifically depicted nucleotide sequences

by virtue of the degeneracy of the genetic code. Also, reference to Figure 1 in the specification and the claims will be understood to encompass both the genomic sequence depicted therein as well as the corresponding cDNA and RNA sequences, and the phrases "DNA construct" and "nucleic acid sequences" as used herein will be understood to encompass all such variations. "DNA construct" shall generally be understood to mean a DNA molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

The *Myceliophthora* laccase described herein has a particularly high specific activity on a syringaldazine substrate relative to other known ascomycete or deuteromycete extracellular laccases in which such specific activity has been described. The present sequence provides a means by which other such ascomycete and/or deuteromycete laccases can also be isolated. Identification and isolation of laccase genes from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the present examples, with publicly available ascomycete and deuteromycete strains. In particular, the specific sequence disclosed herein can be used to design primers and/or probes useful in isolating similar laccase genes by standard PCR or southern hybridization techniques. The present invention thus encompasses those ascomycete and deuteromycete laccases which have a specific activity of at least about 30 SOU/mg, and preferably at least about 40 SOU/mg, "SOU" being defined as  $\mu$ mole of substrate oxidized per minute as measured with syringaldazine as a substrate, at optimum pH.

In addition, the invention also encompasses other *Myceliophthora* laccases, including alternate forms of

laccase which may be found in *M. thermophila* and as well as laccases which may be found in other fungi falling within the definition of *Myceliophthora* as defined by Van Oorschot, 1977, *supra*. Identification and isolation of laccase genes  
5 from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the present examples, with publicly available *Myceliophthora* strains. Alternately, the sequence disclosed herein can be used to design primers and/or probes useful in  
10 isolating laccase genes by standard PCR or southern hybridization techniques. Other named *Myceliophthora* species include *Myceliophthora hinnulea* (Awao et al., Mycotaxon. 16: 436-440, 1983), *Myceliophthora vellerea* (Guarro et al, Mycotaxon. 23: 419-427, 1985), and *Myceliophthora lutea*  
15 Costatin. Also encompassed are laccases which are synonyms, e.g., anamorphs or perfect states of species or strains of the genus *Myceliophthora*. Strains of *Myceliophthora* are readily accessible to the public in a number of culture collections, such as ATCC 48102, 48103, 48104 et al.; CBS  
20 117.65, 131.65, 379.65 et al., DSM 1799 (*M. thermophila*), ATCC 52474, CBS 539.82, 540.82 et al. (*M. hinnulea*), DSM 62114, CBS 146.50, 147.50, 157.51 et al (*M. lutea*), and CBS 478.76, 479.76 and 715.84 (*M. vellerea*). The invention also encompasses any variant nucleotide sequence, and the protein  
25 encoded thereby, which protein retains at least about an 80%, preferably at least 85%, and most preferably at least 90-95% homology with the amino acid sequence depicted in Figure 1, and which qualitatively retains the laccase activity of the sequence described herein. Useful variants  
30 within the categories defined above include, for example, ones in which conservative amino acid substitutions have been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted



by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be determined by conducting a standard ABTS oxidation method, such as is described in the present examples.

The protein can be used in number of different industrial processes. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. A neutral/alkaline laccase is a particular advantage in that Kraft lignin is more soluble at higher pHs. Such methods are described in, for example, Jin et al., *Holzforschung* 45(6): 467-468, 1991; US Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1992.

The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in *Biotechnology* 3: 261-266, 1992; *J. Biotechnol.* 25: 333-339, 1992; Hiroi et al., *Svensk papperstidning* 5: 162-166, 1976. Since the environment in a paper mill is typically alkaline, the present laccase is more useful for this purpose than other known laccases, which function best under acidic conditions.

Oxidation of dyes or dye precursors and other chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 92/01406; WO 92/18683; EP 0495836; Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijksuniversiteit Gent. 56: 1565-1567, 1991; Tsujino et al., J. Soc. Chem. 42: 273-282, 1991.

The laccase is particularly well-suited for use in hair dyeing. In such an application, the laccase is contacted with a dye precursor, preferably on the hair, whereby a controlled oxidation of the dye precursor is achieved to convert the precursor to a dye, or pigment producing compound, such as a quinoid compound. The dye precursor is preferably an aromatic compound belonging to one of three major chemical families: the diamines, aminophenols (or aminonaphthols) and the phenols. The dye precursors can be used alone or in combination. At least one of the intermediates in the copolymerization must be an ortho- or para-diamine or aminophenol (primary intermediate). Examples of such are found in Section IV, below, and include p-phenylene-diamine (PPD), p-toluylene-diamine, chloro-p-phenylenediamine, p-aminophenol, o-aminophenol, 3,4-diaminotoluene; additional compounds are also described in US Patent No. 3,251,742, the contents of which are incorporated herein by reference. In one embodiment, the starting materials include not only the enzyme and a primary intermediate, but also a modifier (coupler) (or combination of modifiers), which modifier is typically a meta-diamine, meta-aminophenol, or a polyphenol. Examples of modifier

compounds include m-phenylene-diamine, 2,4-diaminoanisole,  $\alpha$ -naphthol, hydroquinone, pyrocatechol, resorcinol. and 4-chlororesorcinol. The modifier then reacts with the primary intermediate in the presence of the laccase, converting it to a colored compound. In another embodiment, the laccase can be used with the primary intermediate directly, to oxidize it into a colored compound. In all cases, the dyeing process can be conducted with one or more primary intermediates, either alone or in combination with one or more modifiers. Amounts of components are in accordance with usual commercial amounts for similar components, and proportions of components may be varied accordingly.

The use of this laccase is an improvement over the more traditional use of  $H_2O_2$ , in that the latter can damage the hair, and its use usually requires a high pH, which is also damaging to the hair. In contrast, the reaction with laccase can be conducted at alkaline, neutral or even acidic pH, and the oxygen needed for oxidation comes from the air, rather than via harsh chemical oxidation. The result provided by the use of the *Myceliophthora* laccase is comparable to that achieved with use of  $H_2O_2$ , not only in color development, but also in wash stability and light fastness. An additional commercial advantage is that a single container package can be made containing both the laccase and the precursor, in an oxygen free atmosphere, which arrangement is not possible with the use of  $H_2O_2$ .

The present laccase can also be used for the polymerization of phenolic compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such

applications have been described in Stutz, Fruit processing 7/93, 248-252, 1993; Maier et al., Dt. Lebensmittel-rindschau 86(5): 137-142, 1990; Dietrich et al., Fluss. Obst 57(2): 67-73, 1990,.

- 5 Laccases such as the *Myceliophthora* laccase are also useful in soil detoxification (Nannipieri et al., J. Environ. Qual. 20: 510-517, 1991; Dec and Bollag, Arch. Environ. Contam. Toxicol. 19: 543-550, 1990).

The invention is further illustrated by the following  
10 non-limiting examples.

#### EXAMPLES

##### I. ISOLATION OF MYCELIOPHTHORA THERMOPHILA LACCASE GENE

###### 15 A. MATERIALS AND METHODS

###### 1. DNA Extraction and Hybridization analysis

- Total cellular DNA is extracted from fungal cells of *Myceliophthora thermophila* strain E421 grown 24 hours in 25 ml of YEG medium (0.5% yeast extract, 2% glucose) using the  
20 following protocol: mycelia are collected by filtration through Miracloth (Calbiochem) and washed once with 25 ml of TE buffer. Excess buffer is drained from the mycelia which are subsequently frozen in liquid nitrogen. Frozen mycelia are ground to a fine powder in an electric coffee  
25 grinder, and the powder added to 20 ml of TE buffer and 5 ml of 20% SDS (w/v) in a disposable plastic centrifuge tube. The mixture is gently inverted several times to ensure mixing, and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Sodium acetate  
30 (3M solution) is added to give a final concentration of 0.3 M and the nucleic acids are precipitated with 2.5 volumes of ice cold ethanol. The tubes are centrifuged at 15,000 x g for 30 minutes and the pellet is allowed to air-dry for 30 minutes before resuspending in 0.5 ml of TE buffer. DNase-

free ribonuclease A is added to a concentration of 100µg/ml and the mixture is incubated at 37°C for 30 minutes. Proteinase K (200µg/ml) is added and each tube is incubated an additional one hour at 37°C. Finally, each sample is  
5 extracted twice with phenol:chloroform:isoamyl alcohol before precipitating the DNA with sodium acetate and ethanol. DNA pellets are dried under vacuum, resuspended in TE buffer, and stored at 4°C.

Total cellular DNA samples from transformants and an  
10 untransformed control strain are analyzed by Southern hybridization. Approximately 5µg of DNA is digested with EcoRI and fractionated by size on a 1% agarose gel. The gel is photographed under short wavelength UV and soaked for 15 minutes in 0.5 M NaOH, 1.5 M NaCl followed by 15 minutes in  
15 1 M Tris-HCl, pH 8, 1.5 M NaCl. DNA in the gel is transferred onto Zeta-Probe™ hybridization membrane (BioRad Laboratories) by capillary blotting in 20 X SSPE (R. W. Davis et al., Advanced Bacterial Genetics, A Manual for Genetic Engineering. Cold Spring Harbor Press. 1980)  
20 Membranes are baked for 2 hours at 80°C under vacuum and soaked for 2 hours in the following hybridization buffer at 45°C with gentle agitation: 5X SSPE, 35% formamide (v/v), 0.3% SCS, 200µg/ml denatured and sheared salmon testes DNA. The laccase-specific probe fragment (approx. 1.5 kb)  
25 encoding the 5'-portion of the *N. crassa lcc1* gene is amplified from *N. crassa* genomic DNA using standard PCR conditions (Perkin-Elmer Cetus, Emeryville, CA) with the following pair of primers: forward primer, 5' CGAGACTGATAACTGGCTTGG 3'; reverse primer, 5' ACGGCGCATTTGTCAGGGAAGT 3'.  
30 The amplified DNA segment is first cloned into a TA-cloning vector (Invitrogen, Inc., San Diego, CA), then purified by agarose gel electrophoresis following digestion with EcoRI. The purified probe fragment is radiolabeled by nick translation with α-[<sup>32</sup>P]dCTP (Amersham)

and added to the hybridization buffer at an activity of approximately  $1 \times 10^6$  cpm per ml of buffer. the mixture is incubated overnight at 45°C in a shaking water bath. Following incubation, the membranes are washed once in 0.2 X 5 SSPE with 0.1% SDS at 45°C followed by two washes in 0.2 X SSPE(no SDS) at the same temperature. The membranes are allowed to dry on paper towels for 15 minutes, then wrapped in Saran Wrap™ and exposed to x-ray film overnight at -70°C with intensifying screens(Kodak).

10        2. DNA Libraries and Identification of Laccase Clones

Genomic DNA libraries are constructed in the bacteriophage cloning vector  $\lambda$ -EMBL4(J.A.Sorge, in Vectors, A Survey of Molecular Cloning Vectors and Their Uses, Rodriguez et al., eds, pp.43-60, Butterworths, Boston, 15 1988). Briefly, total cellular DNA is partially digested with *Sau3A* and size-fractionated on low-melting point agarose gels. DNA fragments migrating between 9kb and 23 kb are excised and eluted from the gel using  $\beta$ -agarase (New England Biolabs, Beverly MA). The eluted DNA fragments are 20 ligated with *Bam*HI-cleaved and dephosphorylated  $\lambda$ -EMBL4 vector arms, and the ligation mixtures are packaged using commercial packaging extracts (Stratagene, LaJolla, CA). The packaged DNA libraries are plated and amplified on *Escherichia coli* K802 cells. Approximately 10,000-20,000 25 plaques from each library are screened by plaque-hybridization with the radiolabeled *lcc1* DNA fragment using the conditions described above. Plaques which give hybridization signals with the probe are purified twice on *E. coli* K802 cells, and DNA from the corresponding phage is 30 purified from high titer lysates using a Qiagen Lambda kit(Qiagen, Inc., Chatsworth, CA).

3. Analysis of Laccase Genes

Restriction mapping of laccase clones is done using standard methods (Lewin, Genes. 2d ed., Wiley & Sons, 1985, New York). DNA sequencing is done with an Applied Biosystems Model 373A automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) using the primer walking technique with dye-terminator chemistry (H. Giesecke et al., J. Virol. Methods 38: 47-60, 1992). Oligonucleotide sequencing primers are synthesized on an Applied Biosystems model 394 DNA/RNA Synthesizer.

10

## B. RESULTS AND DISCUSSION

### 1. Identification of Laccase Gene Sequence

Total cellular DNA samples are prepared from the species *Neurospora crassa*, *Botrytis cinerea*, and *Myceliophthora*. Aliquots of these DNA preparations are digested with *Bam*HI and fractionated by agarose gel electrophoresis. DNA in the gel is blotted to a Zeta-Probe™ membrane filter (BioRad Laboratories, Hercules, CA) and probed under conditions of mild stringency with a radiolabeled fragment encoding a portion of the *N. crassa* *lccl* gene, as described above. Laccase-specific sequences are detected in the genomes of *M. thermophila* and the *N. crassa* control, but not in the *B. cinerea* genomic DNA with this probe.

25

### 2. Cloning and Characterization of *Myceliophthora thermophila* Laccase (MtL) Gene

Approximately 20,000 plaques from a *M. thermophila* genomic DNA library constructed in a  $\lambda$ -EMBL4 cloning vector are screened. The library is composed of approximately 10,000 independent clones with inserts ranging in size from 9kb to 23kb. Assuming an average insert size of 10 kb and a total genome size of  $4 \times 10^7$  bp for *M. thermophila*, this figure is about 2.5 times the number of clones required to represent the entire genome. Eight plaques are identified

30

that hybridized strongly to the *N. crassa* laccase gene probe. DNA is isolated from three of these, cleaved with *EcoRI* and analyzed by agarose gel electrophoresis and Southern hybridization. All three of these clones contain a  
5 7.5 kb *EcoRI* fragment which hybridized to the laccase-specific probe. One of these *EcoRI* fragments is subcloned into pBR322 (Bolivar et al., Gene 2: 95-113, 1977) to generate plasmid pRAMB1. A restriction map of this DNA segment is shown in Fig. 1. The position of the laccase  
10 coding region on this clone is determined by hybridization with the *lcc1* gene fragment described above. Based on mapping data obtained, and an estimated size of the laccase protein of approximately 80 kdal, it is reasoned that the entire *M. thermophila* laccase coding region is contained  
15 with a 3.2 kb *NheI*-*BglIII* segment which is then subcloned into pUC119 (Viera and Messing, Methods Enzymol. 153: 3-11, 1987). The nucleotide sequence of this segment is determined using the primer walking method (Giesecke et al., supra). The nucleic acid sequence is shown in Figure 2 and  
20 SEQ ID NO: 1.

The deduced amino acid sequence of MtL is obtained on the basis of amino acid sequence homology with the *N. crassa* laccase. At the amino acid level, these two laccases share approximately 60% sequence identity. Similarity is highest  
25 in regions that correspond to the four histidines and one cysteine which are involved in the formation of the trinuclear copper cluster (Perry et al., J. Gen. Microbiol. 139: 1209-1218, 1993; Coll et al. Appl. Environ. Microbiol. 59: 4129-4135, 1993; Messerschmidt et al. J. Mol. Biol. 206:  
30 513-530, 1989). There are 11 potential sites for N-linked glycosylation in the deduced amino acid sequence of MtL. the first 22 amino acids of MtL appear to comprise a canonical signal peptide with a predicted cleavage following an Ala residue (vonHeijne, J. Mol. Biol. 173:243-251, 1984).



Although the amino terminal sequence of the native MtL is unknown, the amino terminus of recombinant MtL produced in *A. oryzae* is blocked with a pyro-glutamate residue. Enzymatic removal of this residue followed by amino acid sequencing suggests that mature MtL begins with a Gln residue (position 1 in Figure 2; SEQ ID NO: 2). Thus, MtL is apparently synthesized as a 620 amino acid preproenzyme having a 22 amino acid signal peptide and propeptide of 25 residues. *Neurospora crassa* laccase(NcL) is processed similarly at its amino terminal end. In addition, NcL is also proteolytically processed at its C-terminus, resulting in the removal of 13 amino acids (Germann et al. J. Biol. Chem. 263: 885-896, 1988). The processing site is contained within the sequence Asp-Ser-Gly-Leu\*Arg<sub>558</sub> (where \* designates the cleavage site). A similar sequence exists near the C-terminal end of MtL(Asp-Ser-Gly-Leu-Lys<sub>560</sub>), suggesting the *Myceliophthora* enzyme may also be subject to C-terminal processing (Asp-Ser-Gly-Leu\*Lys<sub>560</sub>) which would remove 12 amino acids.

The positions of six introns (85, 84, 102, 72, 147, and 93 nucleotides) within the *lcc1* coding region are determined by comparing the deduced amino acid sequence of MtL to that of NcL and by applying the consensus rules for intron features in filamentous fungi (Gurr et al., in Gene Structure in Eukaryotic Microbes, J.R. Kinghorn, ed.) pp 93-139, IRL Press, Oxford, 1987). The 1860 nucleotides of coding sequence, excluding introns, are rich in guanosine and cytosine (65.5% G+C). The codon usage pattern for this gene reflects the DNA base composition in a strong bias(89.7%) for codons ending in G or C.

## II. EXPRESSION OF MYCELIOPHTHORA LACCASE IN ASPERGILLUS

### A. MATERIALS AND METHODS

#### 1. Bacterial and Fungal Host Strains

*Escherichia coli* JM101 (Messing et al., Nucl. Acids Res. 9:309-321, 1981) is used as a host for construction and routine propagation of laccase expression vectors in this study. Fungal hosts for laccase expression included the  
5 *Aspergillus niger* strains Bo-1, AB4.1 and AB1.13 (Mattern et al., Mol. Gen. Genet. 234: 332-336), as well as a uridine-requiring (*pyrG*) mutant of the  $\alpha$ -amylase-deficient *Aspergillus oryzae* strain HowB104.

## 2. Plasmids

10 Plasmid pRaMB2 is a pUC119 derivative which contains a 3.2 kb *Bgl*II-*Nhe*I fragment of *M. thermophila* genomic DNA encoding MtL. The vector pMWR is constructed by inserting the *A. oryzae* TAKA-amylase promoter and terminator elements from pTAKA17 (Christensen et al., Bio/Technol. 6: 1419-1422,  
15 1988; EP 238 023) into pUC18 (Yanisch-Perron et al., Gene 33: 103-119, 1985). In this vector, there is a unique *Swa*I site at the end of the promoter element and a single *Nsi*I site at the beginning of the terminator for directional cloning of coding sequences. The cloning vehicle pUC518 is derived by  
20 inserting a small linker containing *Nsi*I, *Cla*I, *Xho*I, and *Bgl*II restriction sites between the adjacent *Bam*HI and *Xba*I sites of pUC118 (Vieira and Messing, *supra*). Plasmid pToC68 (WO 91/17243) contains the *A. oryzae* TAKA-amylase promoter and *A. niger glaA* terminator, and pToC90 (WO  
25 91/17243) carries the *A. nidulans amdS* gene.

## 3. Construction of Laccase Expression Vectors

The construction strategy for the laccase expression vector pRaMB5 is outlined in Figure 3. The promoter directing transcription of the laccase gene is obtained from  
30 the *A. oryzae*  $\alpha$ -amylase (TAKA-amylase) gene (Christensen et al., *supra*), as well as the TAKA-amylase terminator region. The plasmid is constructed first by modifying pMWR3 by inserting a small linker which contains an *Apa*I site between

the *SwaI* and *NsiI* sites, creating a plasmid called pMWR3-SAN. *PfuI* polymerase-directed PCR (Stratagene, La Jolla, CA) is used to amplify a short DNA segment encoding the 5'-portion of MtL, from the start codon to an internal *PstI* site (approximately 0.5 kb). The forward primer for this PCR reaction is designed to create an *EcoRI* site just upstream of the start codon. Next, the amplified fragment is digested with *EcoRI* and *PstI* [during this step, the *EcoRI* site is made blunt by treatment with dNTPs and DNA polymerase I (Klenow fragment)] and purified by agarose gel electrophoresis. The 3' portion of the *M. thermophila* coding region is excised from pRAMB2 as a 2kb *PstI*-*ApaI* fragment (this segment also contains approximately 110 bp from the 3'-untranslated region). These two fragments are combined with *SwaI*- and *ApaI*-cleaved pMWR3-SAN in a three-part ligation reaction to generate the laccase expression vector pRAMB5.

#### 4. Transformation of *Aspergillus* host cells

Methods for co-transformation of *Aspergillus* strains are as described in Christensen et al., *supra*. For introduction of the laccase expression vectors into *A. oryzae* HowB 104 *pyrG*, equal amounts (approximately 5 µg each) of laccase expression vector and one of the following plasmids are used: pPYRG (Fungal Genetics Stock Center, Kansas City, KS) which contains the *A. nidulans pyrG* gene (Oakley et al., Gene 61385-399, 1987); pSO2 which harbors the clones *A. oryzae pyrG* gene; pPRYG24 which contains the *A. ficuum* (= *A. niger*) *pyrG* gene. Protrophic (Pyr<sup>+</sup>) transformants are selected on *Aspergillus* minimal medium (Rowlands and Turner, Mol. Gen. Genet. 126: 201-216, 1973), and the transformants are screened for the ability to produce laccase on minimal medium containing 1 mM 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) [ABTS]. Cells which

secrete active laccase oxidize the ABTS, producing a green halo surrounding the colony. Lastly, *A. niger* Bo-1 protoplasts are co-transformed using equal amounts (approximately 5µg each) of laccase expression vector and pToC90 which contains the *A. nidulans amdS* (acetamidase) gene (Hynes et al., Mol. Cell Biol. 3: 1430-1439, 1983. AmdS<sup>+</sup> transformants are selected on Cove minimal medium (Cove, Biochim. Biophys. Acta 113: 51-56, 1966) with 1% glucose as the carbon source and acetamide as the sole nitrogen source and screened for laccase expression on cove medium with 1 mM ABTS.

#### 5. Analysis of Laccase-Producing Transformants

Transformants which produce laccase activity on agar plates are purified twice through conidiospores and spore suspensions in sterile 0.01% Tween-80 are made from each. The density of spores in each suspension is estimated spectrophotometrically ( $A_{595\text{ nm}}$ ). Approximately 0.5 absorbance units of spores are used to inoculate 25 ml of ASPO4 or MY50 medium in 125 ml plastic flasks. The cultures are incubated at 37°C with vigorous aeration (approximately 200 rpm) for four to five days. Culture broths are harvested by centrifugation and the amount of laccase activity in the supernatant is determined using syringaldazine as a substrate. Briefly, 800 µl of assay buffer (25 mM sodium acetate, pH 5.5, 40 µM CuSO<sub>4</sub>) is mixed with 20 µl of culture supernatant and 60 µl of 0.28 mM syringaldazine (Sigma Chemical Co., St. Louis, MO) in 50% ETOH. The absorbance at 530 nm is measured over time in a Genesys 5 UV-vis spectrophotometer (Milton-Roy). One laccase unit(LACU) is defined as the amount of enzyme which oxidizes one µmole of substrate per minute at room temperature. SDS-polyacrylamide gel electrophoresis(PAGE) is done using precast 10-27% gradient gels from Novex(San

Diego, CA). Protein bands are developed using Coomassie Brilliant Blue(Sigma).

## B.RESULTS AND DISCUSSION

### 5        1. Expression of *Myceliophthora* laccase

Laccase-producing transformants are detected by incorporation of ABTS into selective media. Using *pyrG* or *amdS* as the selectable marker, co-transformation frequencies vary from about 30% to 70%. Heterologous expression of MtL  
10 appears to be highest in *A. oryzae* transformants. Furthermore, production appears to be better in ASPO4 medium compared to MY50, although the reasons for this are unknown. SDS-PAGE analysis of culture broth samples shows a prominent laccase band at approximately 80 kdal, which is similar to  
15 the size of the native enzyme purified from *M. thermophila*. Similar analysis of the culture filtrates from *A. niger* Bo-transformants indicate that the laccase band is obscured by very intense glucoamylase and acid-stable amylase protein bands. Results are shown in Table 1.

Table 1. Mtl expression among selected *A. oryzae* and *A. niger* transformants

	HOST STRAIN	TRANSFORMANT	TRANSFORMING DNAS	MTLACU/ML	
				ASPO4	MY50
5	<i>A. oryzae</i> HowB104 <i>pyrG</i>	untransformed	none	0.00	0.00
		RaMB5.15	pRaMB5+pPYRG	0.85	0.29
		RaMB5.30	pRaMB5+pPYRG	0.71	0.87
		RaMB5.33	pRaMB5+pPYRG	0.60	0.26
		RaMB5.108	pRaMB5+PSO2	0.68	0.19
		RaMB5.111	pRaMB5+PSO2	0.70	0.17
		RaMB5.121	pRaMB5+PSO2	0.49	0.20
		RaMB5.142	pRaMB5+PSO2	0.54	0.04
	<i>A. Niger</i> Bo-1	untransformed	none	0.00	0.00
		RaMB5.1	pRaMB5+pToC90	n.d.	0.20
		RaMB5.25	pRaMB5+pToC90	n.d.	0.09
		RaMB5.49	pRaMB5+pToC90	n.d.	0.06
		RaMB5.51	pRaMB5+pToC90	n.d.	0.12
		RaMB5.53	pRaMB5+pToC90	n.d.	0.21
		RaMB5.62	pRaMB5+pToC90	n.d.	0.16

n.d.= not determined

2. Expression in the presence or absence of excess copper

A 1 ml aliquot of a spore suspension of *Aspergillus oryzae* transformant HowB104-pRaMB5.30 (approximately  $10^9$  spores/ml) is added aseptically to a 500 ml shake flask containing 100 ml of sterile shake flask medium (maltose, 50g/l;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2g/l;  $\text{KH}_2\text{PO}_4$ , 10g/l;  $\text{K}_2\text{SO}_4$ , 2g/l;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.5 g/l; Citric acid, 2g/l; yeast extract, 10g/l; trace metals [ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 14.3 g/l;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 2.5 g/l;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.5 g/l;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 13.8 g/l;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 8.5 g/l; citric acid, 3.0 g/l], 0.5 ml/l; urea, 2g/l, made with tap water and adjusted to pH 6.0 before autoclaving), and incubated at 37°C on a rotary shaker at 200 rpm for 18 hours. 50 ml of this culture is aseptically transferred to a 3 liter fermentor containing 1.8 liters of the fermentor media ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2g/l;  $\text{KH}_2\text{PO}_4$ , 2g/l; citric acid 4g/l;  $\text{K}_2\text{SO}_4$ , 3g/l;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2g/l; trace metals, 0.5 ml/l; pluronic antifoam, 1ml/l). The fermentor temperature is maintained at 34°C by the circulation of cooling water through the fermentor jacket. Sterile air is sparged through the fermentor at a rate of 1.8 liter/min (1v/v/m). The agitation rate is maintained between 600 and 1300 rpm at approximately the minimum level required to maintain the dissolved oxygen level in the culture above 20%. Sterile feed (Nutriose 725 [maltose syrup], 225 g/l; urea, 30 g/l; yeast extract, 15 g/l; pluronic antifoam, 1.5 ml/l, made up with distilled water and autoclaved) is added to the fermentor by use of a peristaltic pump. The feed rate profile during the fermentation is as follows: 30 g of feed is added initially before inoculation; 0-24 h, 2 g/l h; 24-48 h, 4 g/l h, 48h-end, 6 g/l.

Copper is made as a 400X stock in water or a suitable buffer, filter sterilized and added aseptically to the tank

to a final level of 0.5 mM. The fermentation described above is also conducted without the addition of copper supplement to the tank medium. Samples for enzyme activity determination are withdrawn and filtered through Miracloth to remove mycelia. These samples are assayed for laccase activity by the LACU assay described above. Laccase activity is found to increase continuously during the course of the fermentation, with a value of approximately 45 LACU/ml achieved after 180 hours in the fermentation containing excess copper. At a specific activity of 22 LACU/mg, this corresponds to 2g/l of recombinant laccase expressed. On the other hand, the maximum laccase activity achieved in the fermentation without copper supplement is approximately 10 LACU/ml after 170 hours, or about 25% of that found in the presence of additional copper.

### III. PURIFICATION AND CHARACTERIZATION OF MYCELIOPHORA LACCASE

#### A. MATERIALS AND METHODS

##### 1. Materials

Chemicals used as buffers and substrates are commercial products of at least reagent grade. Endo/N-glycosidase F and pyroglutamate amino peptidase are purchased from Boehringer Mannheim. Chromatography is performed on either a Pharmacia FPLC or a conventional low pressure system. Spectroscopic assays are conducted on either a spectrophotometer (Shimadzu PC160) or a microplate reader (Molecular Devices). Britton & Robinson (B&R) buffers are prepared according to the protocol described in Quelle, Biochemisches Taschenbuch, H.M. Raven, II. Teil, S.93 u. 102, 1964.

##### 2. Enzymatic Assay

Laccase activity is determined by syringaldazine oxidation at 30°C in a 1-cm quartz cuvette. 60µl



syringaldazine stock solution (0.28 mM in 50% ethanol) and 20  $\mu$ l sample are mixed with 0.8 ml preheated buffer solution. The oxidation is monitored at 530nm over 5 minutes. The activity is expressed as  $\mu$ mole substrate oxidized per minute. B&R buffers with various pHs are used. The activity unit is referred to here as "SOU". A buffer of 25 mM sodium acetate, 40  $\mu$ M CuSO<sub>4</sub>, pH 5.5, is also used to determine the activity, which is referred to as LACU, as defined above. 2,2'-azinobis(3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS) oxidation assays are done using 0.4 mM ABTS, B&R buffer, pH 4.1, at room temperature by monitoring  $\Delta A_{405}$ . An ABTS oxidase activity overlay assay is performed by pouring cooled ABTS-agarose (0.05 g ABTS, 1 g agarose, 50 ml H<sub>2</sub>O, heated to dissolve agarose) over a native IEF gel and incubating at room temperature. Thermostability analysis of the laccase(r-MtL) is performed using samples that have 3 SOU activity pre-incubated in B&R buffer, pH 6, at various temperatures. Samples are assayed after a 400-fold dilution into the same buffer at room temperature.

20     3. Purification from a fermentor broth

3.7 liters of cheese-cloth filtered broth (pH 7.6, 16 mS) is filtered through Whatman #2 filter paper. The broth is concentrated on a Spiral Concentrator (Amicon) with a SLY100 membrane (MWCO:100) from 3700 ml to 200 ml. The concentrate is adjusted to 0.75 mS by diluting it in water and reconcentrated on SLY100 to 170 ml. The washed and concentrated broth has a dense greenish color.

The broth is frozen overnight at -20°C, thawed the next day and loaded onto a Q-sepharose XK26 column (120 ml), pre-equilibrated with 10 mM Tris, pH 7.5, 0.7 mS (Buffer A). The blue laccase band migrates slowing down the column during loading. One group of blue fractions runs through the column after loading and washing by Buffer A. A second group eluted during the linear gradient with Buffer B

(Buffer A plus 2 M NaCl). Some brown material with no laccase activity is eluted out later with 1 M NaOH. SDS-PAGE analysis shows that this preparation results in pure laccase.

5        4. Analyses of amino acid content, extent of glycosylation, and N-terminal sequence

N-terminal sequencing is performed on an ABI 476A sequencer. Total amino acid analysis, from which the extinction coefficient of r-MtL is determined, is performed  
10 on a HP AminoQuant instrument. Deglycosylation is done using endo/N-glucosidase F according to the manufacturer's instructions and carbohydrate content is estimated by mobility difference as determined on SDS-PAGE. N-terminus de-blocking with pyroglutamate amino peptidase is carried  
15 out according to manufacturer's instructions. About 80µg r-MtL is treated with 4 µg peptidase with or without the presence of 1 M urea or 0.1 M guanidine HCl before being blotted on a PVDF membrane for sequencing. About 20 pmol de-blocked protein is obtained and sequenced.

20        SDS-PAGE and native IEF analysis are performed on either a Novex cell or a Mini Protean II and a Model 111 Mini IEF cells (Bio-Rad). Gel filtration analyses are done on a Sephacryl S-300(Pharmacia), from which the native MW is estimated by using Blue Dextran (2000 kdal), bovine IgG (158  
25 kdal), bovine serum albumin (66 kdal), ovalbumin (45 kdal) and horse heart myoglobin(17 kdal) to calibrate the column.

B. RESULTS AND DISCUSSION

1. Purification and characterization of r-MtL from a fermentor broth

30        From 3.7 l of fermentor broth, about 2-3 g of r-MtL are isolated. Initial concentration using a membrane with MWCO of 100 kdal removed significant amounts of brown material and small contaminant proteins. The low affinity of r-MtL toward Q-Sepharose matrix equilibrated with 10 mM Tris, pH

7.5, facilitates its separation from other more acidic and more tightly bound impurities. As shown by SDS-PAGE, this preparation resulted in essentially pure laccase for the most active fractions located around the peak. Other less  
 5 active fractions can be further purified on either Mono-Q with a shallower gradient or a gel filtration column, such as S-300, from which the contaminants are separated due to their smaller MW. An overall 18-fold purification and a recovery of 67% are achieved. As discussed below, the  
 10 existence of two elution bands of r-MtL on Q-Sepharose chromatogram is probably due to a differential glycosylation.

The purified r-MtL shows a MW of 100-140 kdal on S-300 gel filtration and a MW of 85 kdal on SDS-PAGE. The  
 15 increase of r-MtL mobility on SDS-PAGE after deglycosylation suggests that carbohydrates account for 14% of its total mass. Native IEF shows a major band at pI ~4.2 that is active in ABTS overlay assay.

Directly sequencing the N-terminus of the purified r-MtL from samples either in desalted solution or on PVDF  
 20 membrane are unsuccessful. However, treatment of r-MtL with pyroglutamate amino peptidase yielded a protein with deblocked N-terminus. This suggests the processing of a propeptide during the maturation of r-MtL, a  
 25 posttranslational event similar to that of *N. crassa* laccase but not found in other laccases such as *Rhizoctonia solani*. The proposed scheme is outlined below.

MKSFISAATLWIVGILTPSVAAAPPSTEPQRDLLVPITEREEAAVKARQQSCNTPS  
 30 |<-putative signal peptide-> |<- putative propeptide ->|<-N-terminus

The spectrum of the blue r-MtL has absorption maxima at 276 and 589 nm.

The activity of the laccase is tested by using either syringaldazine and ABTS as substrates. Expressed as per Abs<sub>276</sub> or per mg, the laccase has a value of 20 or 45 units for SOU at pH 6.5, respectively. The LACU assay yields a  
5 value of 10 or 22 units per Abs<sub>276</sub> or per mg.

The pH profile of r-MtL activity is quite close to that of the wild type, with an optimal pH of 6.5. The upper temperature limit for retaining full activity after a 20 minute preincubation observed for r-MtL is approximately  
10 60°C. The purified r-MtL shows no activity loss over a 5 week storage frozen in Q-sepharose elution buffer at -20°C.

When comparing the two forms of r-MtL obtained from the fermentor broth isolated on Q-Sepharose, there are no significant differences seen in terms of SDS-PAGE, native  
15 PAGE, native IEF, S-300 gel filtration, UV-visible spectrum, specific activity towards syringaldazine and ABTS, and deblocked N-terminus sequencing measurements. Likely, the different elution pattern on Q-Sepharose arises from some sort of differential glycosylation.

20

#### IV. USE OF MYCELIOPHTHORA LACCASE IN DYEING HAIR

The dyeing effect of *Myceliophthora* laccase is tested on various dye precursors and further on 0.1% p-phenylenediamine compared with a number of modifiers.

#### 25 Materials:

##### Dye precursors:

0.1 % p-phenylene-diamine in 0.1 M K-phosphate buffer, pH=7.0)

0.1 % o-aminophenol in 0.1 M K-phosphate buffer, pH=7.0)

30

##### Enzymes:

Recombinant *Myceliophthora thermophila* laccase, 16 LACU/ml (in final dye solution).

Equipment:

Datascolor Textflash 2000 (CIE-Lab)

Assessment of the hair color

- 5 The quantitative color of the hair tresses is determined on a Datascolor Textflash 2000 by the use of CIE-Lab parameters L\* ("0"=black and "100"=white) combined with a\* ("-"=green and "+"=red).

10 Results:Dyeing effect

- Tresses of blond European hair (1 gram) are used for testing *Myceliophthora thermophila* laccase in the context of oxidative hair dyeing. p-phenylene diamine and o-aminophenol are  
15 used as the dye precursors.

Hair dyeing

- 4 ml dye precursor solution is mixed with 1 ml laccase on a Whirley mixer, applied to the hair tresses and kept at 30°C  
20 for 60 minutes. The hair tresses are then rinsed with running water for about 3 minutes, pressed between two fingers, combed, and air dried.

- The results of the dyeing effect test are displayed below in  
25 Table 1 and 2.

Table 1

o-aminophenol	enzyme	L*	a*
Untreated blond hair	-	70.3	2.3
Laccase	+	57.7	15.3

L\*: 0=black, 100=white    a\*: --green, ++red

30

Table 2

p-phenylenediamine	enzyme	L*	a*
Untreated blond hair	-	70.3	2.3
1.0 ml laccase	+	29.1	4.1

L\*: 0=black, 100=white    a\*: --green, +=red

##### 5 Result of test:

From Table 1 and 2 it can be seen that the *Myceliophthora thermophila* laccase can be used for oxidative dyeing of hair.

##### 10 Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern

- 15 Regional Research Center, 1815 University Street, Peoria, Illinois, 61604 on May 25, 1994, and given the following accession number.

##### Deposit

##### Accession Number

*E. coli* JM101 containing

- 20 pRaMB5

NRRL B-21261

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Novo Nordisk Biotech, Inc.  
(B) STREET: 1445 Drew Avenue  
(C) CITY: Davis, California  
(D) COUNTRY: United States of America  
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(F) TELEPHONE: (916) 757-8100  
(G) TELEFAX: (916) 758-0317

## (i) APPLICANT:

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(B) STREET: Novo Alle  
(C) CITY: Bagsvaerd  
(D) COUNTRY: Denmark  
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(F) TELEPHONE: +45 4444 8888  
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(ii) TITLE OF INVENTION: PURIFIED MYCELIOPHTHORA LACCASES AND NUCLEIC ACIDS ENCFODING SAME

(iii) NUMBER OF SEQUENCES: 2

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Novo Nordisk of North America, Inc.  
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(C) CITY and STATE: New York, New York  
(D) COUNTRY: U.S.A.  
(E) ZIP: 10174-6401

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: to be assigned  
(B) FILING DATE: 31-May-1995  
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: US 08/253,781  
(B) FILING DATE: 03-June-1994

## (viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 31,274  
(C) REFERENCE/DOCKET NUMBER: 4184.204-WO

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 212 867 0123  
(B) TELEFAX: 212 867 0298

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3187 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: *Myceliophthora thermophila*

(ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 833...917

(ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 996...1077

(ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 1090...1188

(ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 1261...1332

(ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 2305...2451

(ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 2521...2613

(ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: join (587..832, 918..995, 1078..1089, 1189..1260, 1333..2304, 2452..2520, 2614..3024)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCTAGCTTCT TTGGTCACCG TCGTTTTCGC CCGCCCCCTC CCTCCTTCAA CCCCCTGAGT	60
AGTCGGCTAA GCGATCCTCA ATCTGGTCTT GTGAGGTCAC GTCCTCCAGC AGATGACAGT	120
TCATCGAGCG AGTGATCTCC ACCACCCAGA AGGGAGGGGG GATGCGCGCA TGCTCCAACA	180
TACCCCTGGTG TCGCTAGAGA CGTCGCGGCA TCAGCCTTTT CATCACACCG AGCACGTCCA	240
CGGACCGGCT CTTTTCACCC CCGCGTCCTC CGGAGGATTG AGTCACGATA TTTCGGGATG	300
TGGGAAGGGG GAGAGAAAGG AGGGGGGAGG GCGGGAACA TGTGATAC GAGCTGCGCC	360
CCTTTTCAA CATCGAGAAC AGGAAGTCGT TGGTGTGGC CGTAATGTC ATAAAACGAG	420
GCTCCTTCTC GTCGTGACT TGTCTCAGGT TCTCTCTC GTCCACACCA AGCCAGTCTT	480
GCCTGAGCCA CTTGAGCCAC CTTCAACTCA TCATCTTCAG TCAAGTCGTT CATTGACATT	540
GTGTCTCTC TTTCTATCGAG TCGGCTTCCC GGCCCTTCAC CACAAC ATG AAG TCC	595
	Met Lys Ser
	1
TTC ATC AGC GCC GCG ACG CTT TTG GTG GGC ATT CTC ACC CCT AGC GTT	643
Phe Ile Ser Ala Ala Thr Leu Leu Val Gly Ile Leu Thr Pro Ser Val	
5 10 15	
GCT GCT GCC CCT CCA TCC ACC CCT GAG CAG CGC GAC CTG CTC GTC CCG	691
Ala Ala Ala Pro Pro Ser Thr Pro Glu Gln Arg Asp Leu Leu Val Pro	
20 25 30 35	



ATC ACG GAG AGG GAG GAG GCA GCC GTG AAG GCT CGC CAG CAG AGC TGC Ile Thr Glu Arg Glu Glu Ala Ala Val Lys Ala Arg Gln Gln Ser Cys 40 45 50	739
AAC ACC CCC AGC AAC CGG GCG TGC TGG ACT GAC GGA TAC GAC ATC AAC Asn Thr Pro Ser Asn Arg Ala Cys Trp Thr Asp Gly Tyr Asp Ile Asn 55 60 65	787
ACC GAC TAC GAA GTG GAC AGC CCG GAC ACG GGT GTT GTT CCG CCG Thr Asp Tyr Glu Val Asp Ser Pro Asp Thr Gly Val Val Arg Pro 70 75 80	832
GTGAGTGCTC TCGTTAATTA CGCTTCGGCG AGTTGCGCAG ATATATTAAA TACTGCAAAC	892
CTAAGCAGGA GCTGACATGC GACAG TAC ACT CTG ACT CTC ACC GAA GTC GAC Tyr Thr Leu Thr Leu Thr Glu Val Asp 85 90	944
AAC TGG ACC GGA CCT GAT GGC GTC GTC AAG GAG AAG GTC ATG CTG GTT Asn Trp Thr Gly Pro Asp Gly Val Val Lys Glu Lys Val Met Leu Val 95 100 105	992
AAC GTACGGCACC CCTTTTCTTG TCCTAGGATC TGGGTGATGT GCGTCGTTGC Asn	1045
CCCTGAGAGA CTGACCGAGC CTTTGGCTGC AG AAT AGT ATA ATC GTAATTAATT Asn Ser Ile Ile 110	1099
ATACCGCCCT GCCTCCAGCA GCCCCAGCAG CTCGAGAAGG GTATCTGAAG TTAGTCAGGC	1159
CTGCTGACCT GACCGGGGCC AACCCATAG GGA CCA ACA ATC TTT GCG GAC TGG Gly Pro Thr Ile Phe Ala Asp Trp 115 120	1212
GGC GAC ACG ATC CAG GTA ACG GTC ATC AAC AAC CTC GAG ACC AAC GGC Gly Asp Thr Ile Gln Val Thr Val Ile Asn Asn Leu Glu Thr Asn Gly 125 130 135	1260
GTATGTCTGC TGCTTGCTCT CTGCTCTCC TCGTCCGCGA CTAATAATAA TATCAACTCG	1320
TGTGAAAAC AG ACG TCG ATC CAC TGG CAC GGA CTG CAC CAG AAG GGC Thr Ser Ile His Trp His Gly Leu His Gln Lys Gly 140 145	1368
ACC AAC CTG CAC GAC GGC GCC AAC GGT ATC ACC GAG TGC CCG ATC CCC Thr Asn Leu His Asp Gly Ala Asn Gly Ile Thr Glu Cys Pro Ile Pro 150 155 160	1416
CCC AAG GGA GGG AGG AAG GTG TAC CGG TTC AAG GCT CAG CAG TAC GGG Pro Lys Gly Gly Arg Lys Val Tyr Arg Phe Lys Ala Gln Gln Tyr Gly 165 170 175 180	1464
ACG AGC TGG TAC CAC TCG CAC TTC TCG GCC CAG TAC GGC AAC GGC GTG Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn Gly Val 185 190 195	1512
GTC GGG GCC ATT CAG ATC AAC GGA CCG GCC TCG CTG CCG TAC GAC ACC Val Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu Pro Tyr Asp Thr 200 205 210	1560
GAC CTG GGT GTG TTC CCC ATC AGC GAC TAC TAC TAC AGC TCG GCC GAC Asp Leu Gly Val Phe Pro Ile Ser Asp Tyr Tyr Ser Ser Ala Asp 215 220 225	1608

GAG Glu 230	CTG Leu	GTG Val	GAA Glu	CTC Leu	ACC Thr 235	AAG Lys	AAC Asn	TCG Ser	GGC Gly	GCG Ala 240	CCC Pro	TTC Phe	AGC Ser	GAC Asp	AAC Asn 245	1656
GTC Val	CTG Leu	TTC Phe	AAC Asn	GGC Gly 250	ACG Thr	GCC Ala	AAG Lys	CAC His	CCG Pro 255	GAG Glu	ACG Thr	GGC Gly	GAG Glu	GGC Gly 260	GAG Glu	1704
TAC Tyr	GCC Ala	AAC Asn	GTG Val 265	ACG Thr	CTC Leu	ACC Thr	CCG Pro	GGC Gly 270	CGG Arg	CGG Arg	CAC His	CGC Arg	CTG Leu 275	CGC Arg	CTG Leu	1752
ATC Ile	AAC Asn	ACG Thr 280	TCG Ser	GTC Val	GAG Glu	AAC Asn	CAC His 285	TTC Phe	CAG Gln	GTC Val	TCG Ser	CTC Leu 290	GTC Val	AAC Asn	CAC His	1800
ACC Thr	ATG Met 295	TGC Cys	ATC Ile	ATC Ile	GCC Ala	GCC Ala 300	GAC Asp	ATG Met	GTG Val	CCC Pro 305	GTC Val	AAC Asn	GCC Ala	ATG Met	ACG Thr	1848
GTC Val 310	GAC Asp	AGC Ser	CTC Leu	TTC Phe	CTC Leu 315	GGC Gly	GTC Val	GGC Gly	CAG Gln	CGT Arg 320	TAC Tyr	GAT Asp	GTC Val	GTC Val	ATC Ile 325	1896
GAA Glu	GCC Ala	AAC Asn	CGA Arg 330	ACG Thr	CCC Pro	GGG Gly	AAC Asn	TAC Tyr	TGG Trp 335	TTT Phe	AAC Asn	GTC Val	ACA Thr	TTT Phe 340	GGC Gly	1944
GGC Gly	GGC Gly	CTG Leu	CTC Leu 345	TGC Cys	GGC Gly	GGC Gly	TCC Ser	AGG Arg 350	AAT Asn	CCC Pro	TAC Tyr	CCG Pro	GCC Ala 355	GCC Ala	ATC Ile	1992
TTC Phe	CAC His	TAC Tyr 360	GCC Ala	GGC Gly	GCC Ala	CCC Pro	GGC Gly 365	GGC Gly	CCG Pro	CCC Pro	ACG Thr	GAC Asp 370	GAG Glu	GGC Gly	AAG Lys	2040
GCC Ala 375	CCG Pro	GTC Val	GAC Asp	CAC His	AAC Asn	TGC Cys 380	CTG Leu	GAC Asp	CTC Leu	CCC Pro	AAC Asn 385	CTC Leu	AAG Lys	CCC Pro	GTC Val	2088
GTG Val 390	GCC Ala	CGC Arg	GAC Asp	GTG Val	CCC Pro 395	CTG Leu	AGC Ser	GGC Gly	TTC Phe	GCC Ala 400	AAG Lys	CGG Arg	GCC Ala	GAC Asp	AAC Asn 405	2136
ACG Thr	CTC Leu	GAC Asp	GTC Val 410	ACC Thr	CTC Leu	GAC Asp	ACC Thr	ACG Thr	GGC Gly 415	ACG Thr	CCC Pro	CTG Leu	TTC Phe	GTC Val 420	TGG Trp	2184
AAG Lys	GTC Val	AAC Asn	GGC Gly 425	AGC Ser	GCC Ala	ATC Ile	AAC Asn	ATC Ile 430	GAC Asp	TGG Trp	GGG Gly	AGG Arg	GCC Ala 435	GTC Val	GTC Val	2232
GAC Asp	TAC Tyr	GTC Val 440	CTC Leu	ACG Thr	CAG Gln	AAC Asn	ACC Thr 445	AGC Ser	TTC Phe	CCA Pro	CCC Pro	GGG Gly 450	TAC Tyr	AAC Asn	ATT Ile	2280
GTC Val 455	GAG Glu	GTG Val	AAC Asn	GGA Gly	GCT Ala	GAT Asp 460	CAG Gln	GTAAGAAAAA	GGGGACCGCA	GGGGTGCTGC						2334
TGCAAGTACA CCTTGCTCGC CCTCCTGTTT TTCCTTAATA ACTACCTCCC AACCTCCCC																2394
CCTAATTAAT TCACTTTAAA GGCCGATCAA GACTGACCGA GCCCCCTCTC TTTGCAG																2451
TGG Trp	TCG Ser	TAC Tyr	TGG Trp 465	TTG Leu	ATC Ile	GAG Glu	AAC Asn	GAT Asp 470	CCC Pro	GGC Gly	GCA Ala	CCT Pro	TTC Phe 475	ACC Thr	CTA Leu	2499

CCG CAT CCG ATG CAC CTG CAC GTAAGTTGGA TACATATATA TATATATATA 2550  
Pro His Pro Met His Leu His  
480

TACATTGCTT TCCTGGCTCG CTCCCTTAAA TAAATTTAAA TAACCAAAAA TAACAAAAAA 2610

AAG GGC CAC GAC TTT TAC GTG CTG GGC CGC TCG CCC GAC GAG TCG CCG 2658  
Gly His Asp Phe Tyr Val Leu Gly Arg Ser Pro Asp Glu Ser Pro  
485 490 495

GCA TCC AAC GAG CGG CAC GTG TTC GAT CCG GCG GAC GCG GGC CTG 2706  
Ala Ser Asn Glu Arg His Val Phe Asp Pro Ala Arg Asp Ala Gly Leu  
500 505 510 515

CTG AGC GGG GCC AAC CCT GTG CGG CGG GAC GTG TCG ATG CTG CCG GCG 2754  
Leu Ser Gly Ala Asn Pro Val Arg Arg Asp Val Ser Met Leu Pro Ala  
520 525 530

TTC GGG TGG GTG GTG CTG TCC TTC CGG GCC GAC AAC CCG GGC GCC TGG 2802  
Phe Gly Trp Val Val Leu Ser Phe Arg Ala Asp Asn Pro Gly Ala Trp  
535 540 545

CTG TTC CAC TGC CAC ATC GCC TGG CAC GTC TCG GGC GGC CTG GGC GTC 2850  
Leu Phe His Cys His Ile Ala Trp His Val Ser Gly Gly Leu Gly Val  
550 555 560

GTC TAC CTC GAG CGC GCC GAC GAC CTG CGC GGG GCC GTC TCG GAC GCC 2898  
Val Tyr Leu Glu Arg Ala Asp Asp Leu Arg Gly Ala Val Ser Asp Ala  
565 570 575

GAC GCC GAC GAC CTC GAC CGC CTC TGC GCC GAC TGG CGC CGC TAC TGG 2946  
Asp Ala Asp Asp Leu Asp Arg Leu Cys Ala Asp Trp Arg Arg Tyr Trp  
580 585 590

CCT ACC AAC CCC TAC CCC AAG TCC GAC TCG GGC CTC AAA CAC CGC TGG 2994  
Pro Thr Asn Pro Tyr Pro Lys Ser Asp Ser Gly Leu Lys His Arg Trp  
595 600 605

GTC GAG GAG GGC GAG TGG CTG GTC AAG GCG TGAGCGAAGG AGGAAAAAGG 3044  
Val Glu Glu Gly Glu Trp Leu Val Lys Ala  
610 615

AAACAAAGAG GGGGGGGGGG GCTAGTTTCCT ATTTTTCCTT TTTTTCCTTG TTCTTGTCTT 3104

TGTGCTGGCG GTTCCCTGGT AAAGGAGAAG GGGGCCCCAA GTTCGAGTGG GTGTGTGATC 3164

GGGTAAATAT TATCAAGAGA TCT 3187

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 620 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Myceliophthora thermophila

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Ser Phe Ile Ser Ala Ala Thr Leu Leu Val Gly Ile Leu Thr  
1 5 10 15

Pro Ser Val Ala Ala Ala Pro Pro Ser Thr Pro Glu Gln Arg Asp Leu  
 20 25 30  
 Leu Val Pro Ile Thr Glu Arg Glu Glu Ala Ala Val Lys Ala Arg Gln  
 35 40 45  
 Gln Ser Cys Asn Thr Pro Ser Asn Arg Ala Cys Trp Thr Asp Gly Tyr  
 50 55 60  
 Asp Ile Asn Thr Asp Tyr Glu Val Asp Ser Pro Asp Thr Gly Val Val  
 65 70 75 80  
 Arg Pro Tyr Thr Leu Thr Leu Thr Glu Val Asp Asn Trp Thr Gly Pro  
 85 90 95  
 Asp Gly Val Val Lys Glu Lys Val Met Leu Val Asn Asn Ser Ile Ile  
 100 105 110  
 Gly Pro Thr Ile Phe Ala Asp Trp Gly Asp Thr Ile Gln Val Thr Val  
 115 120 125  
 Ile Asn Asn Leu Glu Thr Asn Gly Thr Ser Ile His Trp His Gly Leu  
 130 135 140  
 His Gln Lys Gly Thr Asn Leu His Asp Gly Ala Asn Gly Ile Thr Glu  
 145 150 155 160  
 Cys Pro Ile Pro Pro Lys Gly Gly Arg Lys Val Tyr Arg Phe Lys Ala  
 165 170 175  
 Gln Gln Tyr Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr  
 180 185 190  
 Gly Asn Gly Val Val Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu  
 195 200 205  
 Pro Tyr Asp Thr Asp Leu Gly Val Phe Pro Ile Ser Asp Tyr Tyr Tyr  
 210 215 220  
 Ser Ser Ala Asp Glu Leu Val Glu Leu Thr Lys Asn Ser Gly Ala Pro  
 225 230 235 240  
 Phe Ser Asp Asn Val Leu Phe Asn Gly Thr Ala Lys His Pro Glu Thr  
 245 250 255  
 Gly Glu Gly Glu Tyr Ala Asn Val Thr Leu Thr Pro Gly Arg Arg His  
 260 265 270  
 Arg Leu Arg Leu Ile Asn Thr Ser Val Glu Asn His Phe Gln Val Ser  
 275 280 285  
 Leu Val Asn His Thr Met Cys Ile Ile Ala Ala Asp Met Val Pro Val  
 290 295 300  
 Asn Ala Met Thr Val Asp Ser Leu Phe Leu Gly Val Gly Gln Arg Tyr  
 305 310 315 320  
 Asp Val Val Ile Glu Ala Asn Arg Thr Pro Gly Asn Tyr Trp Phe Asn  
 325 330 335  
 Val Thr Phe Gly Gly Gly Leu Leu Cys Gly Gly Ser Arg Asn Pro Tyr  
 340 345 350  
 Pro Ala Ala Ile Phe His Tyr Ala Gly Ala Pro Gly Gly Pro Pro Thr  
 355 360 365

Asp Glu Gly Lys Ala Pro Val Asp His Asn Cys Leu Asp Leu Pro Asn  
 370 375 380  
 Leu Lys Pro Val Val Ala Arg Asp Val Pro Leu Ser Gly Phe Ala Lys  
 385 390 400  
 Arg Ala Asp Asn Thr Leu Asp Val Thr Leu Asp Thr Thr Gly Thr Pro  
 405 410 415  
 Leu Phe Val Trp Lys Val Asn Gly Ser Ala Ile Asn Ile Asp Trp Gly  
 420 425 430  
 Arg Ala Val Val Asp Tyr Val Leu Thr Gln Asn Thr Ser Phe Pro Pro  
 435 440 445  
 Gly Tyr Asn Ile Val Glu Val Asn Gly Ala Asp Gln Trp Ser Tyr Trp  
 450 455 460  
 Leu Ile Glu Asn Asp Pro Gly Ala Pro Phe Thr Leu Pro His Pro Met  
 465 470 475 480  
 His Leu His Gly His Asp Phe Tyr Val Leu Gly Arg Ser Pro Asp Glu  
 485 490 495  
 Ser Pro Ala Ser Asn Glu Arg His Val Phe Asp Pro Ala Arg Asp Ala  
 500 505 510  
 Gly Leu Leu Ser Gly Ala Asn Pro Val Arg Arg Asp Val Ser Met Leu  
 515 520 525  
 Pro Ala Phe Gly Trp Val Val Leu Ser Phe Arg Ala Asp Asn Pro Gly  
 530 535 540  
 Ala Trp Leu Phe His Cys His Ile Ala Trp His Val Ser Gly Gly Leu  
 545 550 555 560  
 Gly Val Val Tyr Leu Glu Arg Ala Asp Asp Leu Arg Gly Ala Val Ser  
 565 570 575  
 Asp Ala Asp Ala Asp Asp Leu Asp Arg Leu Cys Ala Asp Trp Arg Arg  
 580 585 590  
 Tyr Trp Pro Thr Asn Pro Tyr Pro Lys Ser Asp Ser Gly Leu Lys His  
 595 600 605  
 Arg Trp Val Glu Glu Gly Glu Trp Leu Val Lys Ala  
 610 615 620

What we claim is:

1. A DNA construct containing a sequence encoding a *Myceliophthora* laccase.
- 5 2. The construct of Claim 1 which comprises a sequence encoding a *Myceliophthora thermophila* laccase.
3. The construct of Claim 1 which comprises a nucleic acid  
10 sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.
4. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.
- 15 5. The construct of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21261.
6. A substantially pure *Myceliophthora* laccase enzyme.
- 20 7. The enzyme of Claim 6 which is a *Myceliophthora thermophila* laccase.
8. The enzyme of Claim 6 which comprises the amino acid  
25 sequence depicted in SEQ ID NO. 2, or a sequence with at least about 80% homology thereto.
9. A recombinant vector comprising an DNA construct containing a sequence encoding a *Myceliophthora* laccase.
- 30 10. The vector of Claim 9 in which the construct is operably linked to a promoter sequence.

11. The vector of Claim 10 in which the promoter is a fungal or yeast promoter.
12. The vector of Claim 11 in which the promoter is the  
5 TAKA amylase promoter of *Aspergillus oryzae*.
13. The vector of Claim 11 in which the promoter is the glucoamylase (gluA) promoter of *Aspergillus niger* or *Aspergillus awamori*.
- 10 14. The vector of Claim 9 which also comprises a selectable marker.
- 15 15. The vector of Claim 14 in which the selectable marker is selected from the group consisting of *amdS*, *pyrG*, *argB*, *niaD*, *sc*, and *hygB*.
16. The vector of Claim 14 in which the selectable marker is the *amdS* marker of *Aspergillus nidulans* or *Aspergillus*  
20 *oryzae*, or the *pyrG* marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*.
17. The vector of Claim 14 which comprises both the TAKA  
25 amylase promoter of *Aspergillus oryzae* and the *amdS* or *pyrG* marker of *Aspergillus nidulans* or *Aspergillus oryzae*.
18. A recombinant host cell comprising a heterologous nucleic acid construct containing a nucleic acid sequence  
30 encoding a *Myceliophthora laccase*.
19. The cell of Claim 18 which is a fungal cell.
20. The cell of Claim 19 which is an *Aspergillus* cell.

21. The cell of Claim 18 in which the construct is integrated into the host cell genome.
- 5 22. The cell of Claim 18 in which the construct is contained on a vector..
23. The cell of Claim 18 which comprises a construct containing a sequence encoding the amino acid sequence depicted in SEQ ID NO. 2..
- 10 24. A method for obtaining a laccase enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Myceliophthora* laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.
- 15 25. A *Myceliophthora* enzyme obtained by the method of Claim 24.
- 20 26. An ascomycete or deuteromycete laccase having a specific activity of at least 30 SOU/mg on syringaldazine at optimum pH.
- 25 27. A method of enhancing yield of active recombinant copper-containing enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a sequence encoding a copper containing enzyme, under conditions conducive to expression of the enzyme, in the presence of at least about 0.02mM copper.
- 30 28. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Myceliophthora* laccase.



29. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Myceliophthora* laccase.
- 5 30. A method for oxidizing dyes or dye precursors which comprises contacting the dye with a *Myceliophthora* laccase.
31. A method for dyeing hair which comprises contacting a  
10 *Myceliophthora* laccase, in the presence or absence of at least one modifier, with at least one dye precursor, for a time and under conditions sufficient to permit oxidation of the dye precursor to a dye.
- 15 32. The method of claim 31 in which the dye precursor is selected from the group consisting of a diamine, aminophenol, and a phenol.
33. The method of claim 31, wherein the modifier, when  
20 used, is a meta-diamine, a meta-aminophenol or a polyphenol.
34. The method of claim 32 in which the dye precursor is a primary intermediate selected from the group consisting of an ortho- or para-diamine or aminophenol.
- 25 35. The method of claim 31 in which more than one dye precursor is used.
36. The method of claim 31 in which more than one modifier  
30 is used.
37. The method of claim 31 in which both a primary intermediate and a modifier are used.

38. A dye composition comprising a *Myceliophthora* laccase  
combined with at least one dye precursor.
39. A dye composition comprising a *Myceliophthora* laccase  
5 combined with at least one primary intermediate and at least  
one modifier.
40. A container containing a dye composition comprising a  
*Myceliophthora* laccase and at least one dye precursor in an  
10 oxygen-free atmosphere.
41. The container of claim 40 which contains at least one  
primary intermediate dye precursor combined with at least one  
modifier.
- 15 42. A method of polymerizing or oxidizing a phenolic or  
aniline compound which comprises contacting the phenolic or  
aniline compound with a *Myceliophthora* laccase.

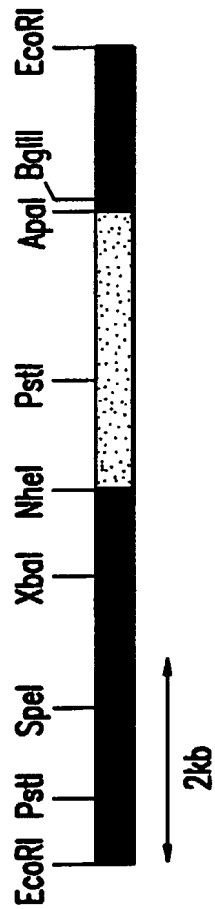


FIG.1

gctagcttcttggtcaccgctgtttcgccgccccctccctccttcaacccctgagtagtcggctlaagcgatcctcg 80  
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 gatgcgcgatgctccaacataccctgggtgctcgtagogacgctcgggcatcagccttttcatcacaccgagcacgtcca 240  
 cggaccggctcctttcaccgccgctcctccggaggattgagtcacgatattcgggatgtgggaaggggagagagaagg 320  
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 MetLysSerPheIleSerAlaAlaThrLeuLeuValGlyIleLeuThrProSer -30  
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 ValAlaAlaAlaProProSerThrProGluGlnArgAspLeuLeuValProIleThrGluArgGluGluAlaAlaValLy -3  
 GGCTCGCCAGCAGAGCTGCAACACCCCCAGCAACCGGGCGTGGACTGACGGATAACGACATCAACACCGACTACGAAG 800  
 sAlaArgGlnGlnSerCysAsnThrProSerAsnArgAlaCysTrpThrAspGlyTyrAspIleAsnThrAspTyrGluV 25  
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 lyProT 68  
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FIG.2A

ctctcttgcctcctcgtccgcgaactaataaataatcaactcgtlggaaaacagCACGTCGATCCACTGGCACGGACT 1360  
 yThrSerIleHisTrpHisGlyLe 97

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 uHisGlnLysGlyThrAsnLeuHisAspGlyAlaAsnGlyIleThrGluCysProIleProProLysGlyGlyArgLysV 124

TGTACGGTTCAAGGCTCAGCAGTACGGGACGAGCTGGTACCACTCGCACTTCTCGCCCACTACGGCAACGGCGTGGTC 1520  
 alTyrArgPheLysAlaGlnGlnTyrGlyThrSerTrpTyrHisSerHisPheSerAlaGlnTyrGlyAsnGlyValVal 150

GGGGCCATTGATCAACGGACCGGCTCGCTGCCGTACGACACCGACCTGGGTGTGTTCCCATCAGCGACTACTACTA 1600  
 GlyAlaIleGlnIleAsnGlyProAlaSerLeuProTyrAspThrAspLeuGlyValPheProIleSerAspTyrTyrTy 177

CAGCTCGGCGACGAGCTGGTGAACCTACCAAGAACTCGGGCGGCCCTTCAGCGACAACGTCCTGTTCAACGGCACGG 1680  
 rSerSerAlaAspGluLeuValGluLeuThrLysAsnSerGlyAlaProPheSerAspAsnValLeuPheAsnGlyThrA 204

CCAAGCACCGGAGACGGGGGAGGGCGAGTACGCCAACGTGACGCTACCCCGGGCGGGCGCACCGCTGCGCCTGATC 1760  
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AACACGTCGGTCGAGAACCCTTCCAGGTCTCGCTCGTCAACCACACCATGTGCATCATCGCCGCGACATGGTGCCCGT 1840  
 AsnThrSerValGluAsnHisPheGlnValSerLeuValAsnHisThrMetCysIleIleAlaAlaAspMetValProVa 257

CAACGCCATGACGGTCGACAGCCTCTTCTCGGGCTCGGCCAGCGTTACGATGTGTCATCGAAGCCAACCGAACGCGCG 1920  
 lAsnAlaMetThrValAspSerLeuPheLeuGlyValGlyGlnArgTyrAspValValIleGluAlaAsnArgThrProG 284

GGACTACTGGTTTAAAGTCACATTTGGCGGGCGCTGCTCTGGGGGGCTCCAGGAATCCCTACCGGGCGGCCATCTTC 2000  
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CACTACG 2080  
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CCTCAAGCCCGTCTGTCG 2160  
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ACACCACGGGCAACCGCCTGTTCTGCTGGAAGGTCAACGGCAGCGCCATCAACATCGACTGGGGGAGGGCGCTCGTGAC 2240  
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 TrpSerTyrTrpLeuIleGluAsn 421

FIG.2B

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SUBSTITUTE SHEET (RULE 26)

GATCCCGGCGCACCTTTACCCCTACCGCATCCGATGCACCTGCACglaagltggalacatalatatatatatacat 2560  
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 AlaAsnProValArgArgAspValSerMetLeuProAlaPheGlyTrpValValLeuSerPheArgAlaAspAsnProG 497  
  
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FIG.2C

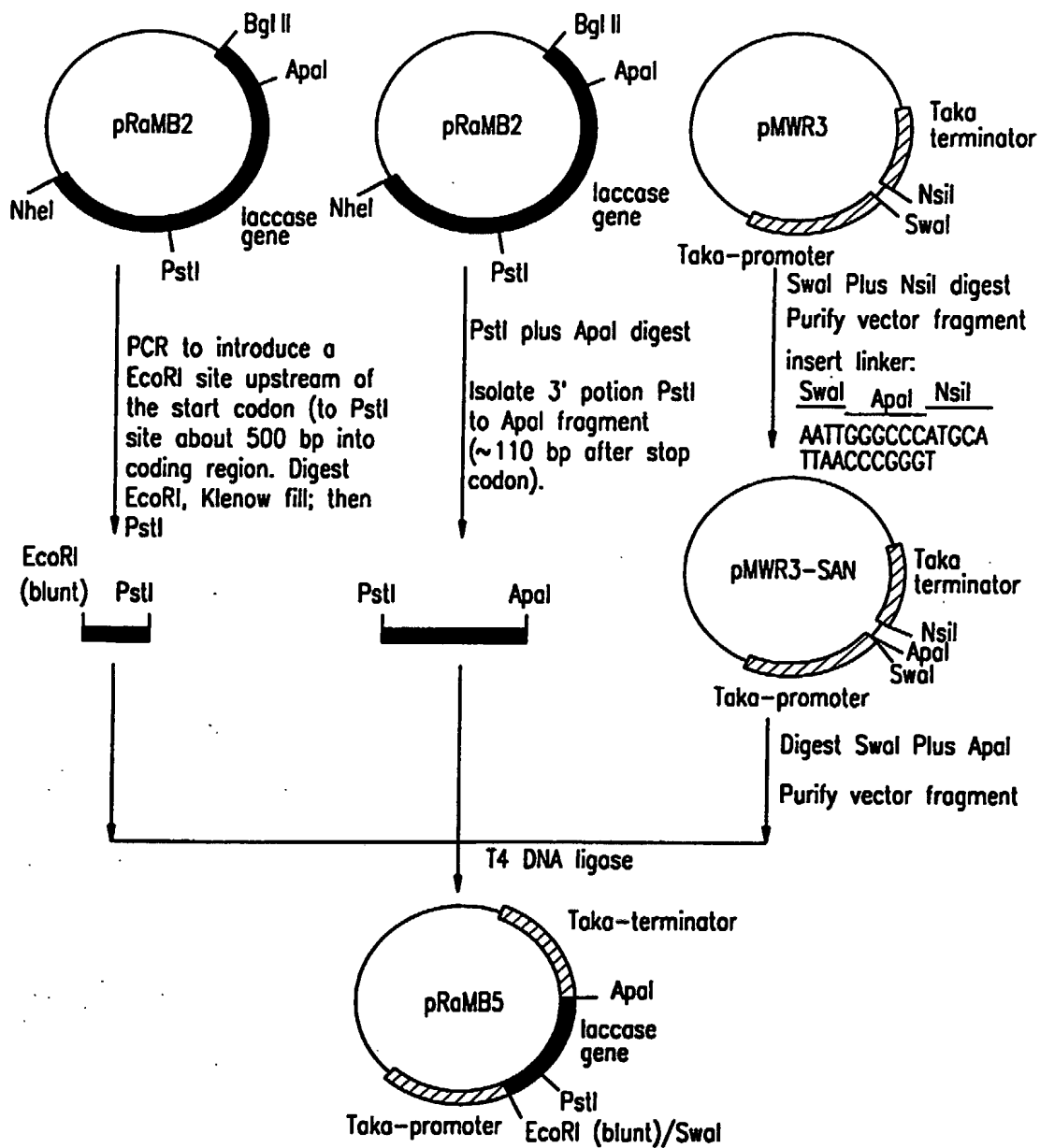


FIG.3

# INTERNATIONAL SEARCH REPORT

Intern: J Application No  
PCT/US 95/06815

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N15/53 C12N1/15 A61K7/13 A61K7/06 D21C5/00 //(C12N1/15,C12R1:66)		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A61K D21C		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P.O. X	ABSTRACTS OF PAPERS, vol.209, no.1-2, April 1995, ANAHEIM, CA BERKA R. ET AL. 'Cloning of laccases from the thermophilic fungi Myceliophthora thermophila and Scytalidium thermophilum and their heterologous expression in Aspergillus oryzae' see BIOT 196	1,2,6,7, 9,10, 18-20, 24-26
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.263, no.2, 1988, BALTIMORE, MD US pages 885 - 896 GERMANN U. ET AL. 'Characterization of two allelic forms of Neurospora crassa laccase' see page 885, right column <div style="text-align: center;">--- -/-- ---</div>	26
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"B" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <div style="text-align: center;">29 August 1995</div>		Date of mailing of the international search report  <div style="text-align: center;">11. 09. 95</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Authorized officer  <div style="text-align: center;">Espen, J</div>



# INTERNATIONAL SEARCH REPORT

Intern. Application No.  
PCT/US 95/06815

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 317 243 (KYOWA MEDEX CO. LTD.) 24 May 1989	
P,X	WO,A,95 01426 (NOVO NORDISK A/S) 12 January 1995 see page 19; claim 22	6,7,25, 26

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/US 95/06815

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0317243	24-05-89	JP-A- 1128797	22-05-89
		DE-D- 3851304	06-10-94
		DE-T- 3851304	26-01-95
		US-A- 5196312	23-03-93
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WO-A-9501426	12-01-95	AU-B- 6924594	24-01-95
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